Virology 410 (2011) 56–63

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

# Reduced innate immune response, apoptosis, and virus release in cells cured of respiratory syncytial virus persistent infection

Cristina Herranz [a], José A. Melero [b,c], Isidoro Martínez [a,b,*]

[a] Unidad de Interacción Virus-Célula, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain
[b] Centro de Investigación Biomédica en Red, Enfermedades Respiratorias (CIBERES), Instituto de Salud Carlos III, Madrid, Spain
[c] Unidad de Biología Viral, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain

ARTICLE INFO

*Article history:*
Received 30 July 2010
Returned to author for revision
25 August 2010
Accepted 24 October 2010
Available online 18 November 2010

*Keywords:*
Respiratory syncytial virus
Innate immune response
Apoptosis
Toll-like receptor
RIG-I
Chemokine
Interleukin
Virus persistence

ABSTRACT

It has been reported that cell clones isolated at different passages from a culture of HEp-2 cells infected persistently with human respiratory syncytial virus (HRSV) were cured of the virus. Further studies on one of these clones (31C1) are reported here, showing that 31C1 cells can still be infected by HRSV but release low amounts of virus to the culture supernatant, develop smaller and less numerous syncytia than the original HEp-2 cells, and display only a weak innate immune response to the infection. Accordingly, uninfected 31C1 cells, but not clones derived from uninfected HEp-2 cells, express low levels of TLR3 and RIG-I. In addition, 31C1 cells are partly resistant to apoptosis. These results indicate that persistent infection of HEp-2 cells by HRSV has selected cell variants, with changes affecting cell survival, virus growth and the innate immune response that may be valuable for studies of virus–cell interaction.



## Introduction

Human respiratory syncytial virus (HRSV) (genus Pneumovirus, family Paramyxoviridae) infects epithelial cells of the lower respiratory tract leading to serious illnesses, such as bronchiolitis and pneumonia, in infants and in the elderly (Collins and Crowe, 2007). Infection in early life increases the probability of subsequent long-term episodes of wheezing and asthma (Perez-Yarza et al., 2007). HRSV infection has also been associated with adult chronic obstructive pulmonary disease (COPD) (Sikkel et al., 2008). In all cases, an inadequate immune/inflammatory response seems to contribute to development of those pathologies (Collins and Graham, 2008; Oshansky et al., 2009). Thus, along with virus replication, the immune/inflammatory response is a major target for prophylactic and/or therapeutic measures to control HRSV infection. The unfortunate outcome of the vaccination trial conducted in the early 1960s, in which children vaccinated with formalin-inactivated virus experienced an enhanced disease upon natural subsequent HRSV infection, has also boosted studies oriented to understand the relationship between HRSV associated pathology and the immune response (Kapikian et al., 1969; Prince et al., 2001).

The host response to HRSV infection begins in the epithelial cells of the respiratory tract, where virus replication preferentially occurs (Krishnan et al., 2004). These cells secrete chemokines and other immune factors that attract lymphocytes and other cells to the site of infection resulting in airway inflammation. Studies of the innate immune response elicited in infected epithelial cells are, therefore, instrumental to understand the pathology caused by HRSV.

Although HRSV normally results in lytic infections in humans, model animals or susceptible cells, there is circumstantial evidence that this virus can persist in the airways of laboratory animals (Hegele et al., 1994; Schwarze et al., 2004; Streckert et al., 1996), or humans (Sikkel et al., 2008; Wilkinson et al., 2006). Furthermore, susceptible cells, such as HEp-2, which survive a lytic infection, become persistently infected with HRSV, resulting in an attractive model for studies of the innate immune response to the virus (Martinez et al., 2009)

We have described previously the establishment of a culture of HEp-2 cells persistently infected with HRSV. This culture turned out to be an extremely complex example of virus–cell interaction in which virus was produced at a relatively high constant level despite heterogeneity among cells in viral antigen production, ranging from high to undetectable levels. However, cell clones derived from early passages (passage 4) of the persistently infected culture were

* Corresponding author. Unidad de Interacción Virus-Célula, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, 28220 Madrid, Spain. Fax: +34 91 8223269.
*E-mail address:* imago@isciii.es (I. Martínez).

0042-6822/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2010.10.035

apparently "cured" since neither virus nor RNA or viral antigens were detected (Martinez et al., 2009). These characteristics were also observed in clones from later passages (passages 15 and 31).

We now report the characterization of a "cured" cell clone (31C1), isolated after 31 passages of the persistently infected HEp-2 culture. The aim behind this study was to assess if the persistent infection may have selected cells with altered expression of genes relevant for virus growth, cell survival and/or innate immune response. The selected clone produced neither infectious virus, viral antigens or RNA, was resistant to UV-induced apoptosis, and showed an impaired innate immune response and decreased virus release when infected with wild-type HRSV. Virus persistence seems to be a key factor for selection of this cell phenotype since clones from the original, uninfected cells did not show those changes.

## Results

### *Cytopathic effect, virus replication and virus release in 31C1 cells infected with HRSV*

A culture of HEp-2 cells infected persistently with HRSV was reported in which virus production was maintained constant for more than twenty passages and a high heterogeneity was found at the cell level regarding viral antigen expression (Martinez et al., 2009). Cell clones derived from this culture were apparently cured of HRSV, since they showed no morphological signs of infection, did not produce detectable virus, were negative for HRSV antigens and no product was amplified by RT-PCR with primers that readily amplified a conserved region of the viral nucleoprotein gene in the persistently infected cells (not shown). All clones were, however, susceptible to HRSV infection, although virus production was lower than in the original HEp-2 cells and syncytia were rarely observed. Since every cell clone obtained at different passages of the persistent culture shared these characteristics, one of them, obtained from the thirty first passage (31C1), was chosen for further studies, based on the hypothesis that the longer the time the cells had been in the presence of the virus, the higher the probability that changes were selected that might be relevant to studies of HRSV–cell interaction. The 31C1 cells showed the above-mentioned characteristics, including the development of smaller and less numerous syncytia than HEp-2 cells following HRSV infection (Fig. 1A). Although infected 31C1 cells died eventually, large syncytia were not observed at any time; instead, rounding up of cells detached from the plates and floating in the culture supernatants was the most prominent effect observed at later times (48–72 h) after infection (not shown). Furthermore, the amount of virus released to the supernatant was approximately forty times lower in 31C1 cells by 20 h p.i., when virus was growing exponentially (Fig. 1B). It has been reported previously that a large proportion of the infectivity of HRSV remains cell-associated in infected cultures (Bachi, 1988; Levine and Hamilton, 1969). Here, we confirmed that in infected HEp-2 cells about ten times more virus was recovered from the infected cells, after disaggregation and brief sonication, than from the supernatant. In infected 31C1 cells, however, the difference between the amount of virus recovered from the infected cells and the supernatant was much higher, so that the amount of cell-associated virus reached values only slightly lower than those obtained from infected HEp-2 cultures (Fig. 1B). To test if these observations could be extended to other viruses, similar experiments were conducted using influenza and vesicular stomatitis virus (VSV). No apparent differences in the cytophatic effect were observed between HEp-2 and 31C1 cells after influenza or VSV virus infection (not shown), but, in both cases, virus production was higher in 31C1 cells, both in the supernatant and in the cell-associated fraction (Fig. 1B).

The results from HRSV infections suggest that virus replication was apparently similar in both HEp-2 and 31C1 cells, but that virus release was diminished in the later. To confirm this point, HRSV replication was monitored at early times p.i. by quantitative RT-PCR of a region of the nucleoprotein gene. The kinetics of RNA accumulation in both types of cells was indistinguishable within the first 9 h of infection (Fig. 1C, left). In addition, three independent infections were carried out in order to monitor differences in the accumulation of viral RNA at 20 h p.i. By this time, when the amount of virus shed to the supernatant was about forty times less in 31C1 cells than in HEp-2 cells, there were almost no differences in the amount of viral RNA, estimated by quantitative RT-PCR (Fig. 1C, right).

The impairment in syncytia formation and virus release in 31C1 cells indicated that HRSV spreading was less efficient in these cells than in HEp-2. Flow cytometry of HEp-2 and 31C1 cells infected with HRSV at high and low MOI also supported this conclusion. Thus, when a MOI of 5 pfu/cell was used, a single peak of fluorescence was found in the two types of cells. However, when a MOI ten times lower was used for infection, two major peaks of fluorescence were seen (Fig. 2). One of them matched with the peak from MOI 5, and corresponded presumably to cells infected by the inoculum. The other peak was less intense and originated probably from a second round of infection with virus released from the initially infected cells. The fluorescence of the first peak was similar in HEp-2 and 31C1 cells, however the fluorescence of the second peak was less intense in 31C1 cells (Fig. 2) than in HEp-2, reflecting reduced spreading of the infection in the former cells.

### *31C1 cells are partially resistant to apoptosis*

It has been previously reported that the persistently infected HEp-2 cells, from which 31C1 cells derived, are partly resistant to apoptosis (Martinez et al., 2009). This might be either an inherent property of the persistent infection itself, or an intrinsic property selected in the cells that survive the infection. To clarify this point, 31C1 cells were irradiated with UV light and fragmentation of DNA was analyzed by agarose gel electrophoresis (Fig. 3A) and ELISA (Fig. 3B). Both techniques gave similar results; 31C1 cells were more resistant than HEp-2 cells to fragmentation of DNA induced by UV light. Quantitative data from the ELISA test gave a difference of more than six times in the amount of fragmented DNA between both cell types (Fig. 3B).

### *The innate immune response is impaired in 31C1 cells*

HRSV infection elicits a strong innate immune response in many cell types, including HEp-2 (Martinez et al., 2009; Zhang et al., 2001). Since 31C1 cells, or rather the precursors from which they originate, had been in contact with HRSV for many passages, we reasoned that this passage history might have selected for cells with a modified innate immune response to the virus. Thus, the innate immune responses of 31C1 and HEp2-cells to HRSV infection were compared at three different levels: expression of pattern recognition receptors (PRRs), activation of transcription factors, and secretion of cytokines (Fig. 4).

Since PRRs are essential factors in detecting viral infection and triggering of the antiviral innate immune response, the expression of TLR4, TLR3 and RIG-1, three PRRs involved in the innate response against HRSV in epithelial cells (Klein Klouwenberg et al., 2009), were quantified by real-time RT-PCR in uninfected HEp-2 and 31C1 cells. Whereas the amount of mRNA corresponding to TLR4 was almost equal in both cell types, the amount of TLR3 and RIG-I was approximately twenty-five and eleven times lower in 31C1 than in HEp-2 cells, respectively (Fig. 4A). These results suggested that the innate immune response of 31C1 cells to HRSV infection, mediated by those two PRRs, could be substantially weaker in 31C1 than in HEp-2 cells. If this were indeed the case, it would be likely to affect the expression and activation of key transcription factors involved in that response. Activation of NF-κB, as seen by degradation of IKBα, and induction of c-Jun were evident after infection of HEp-2 cells with

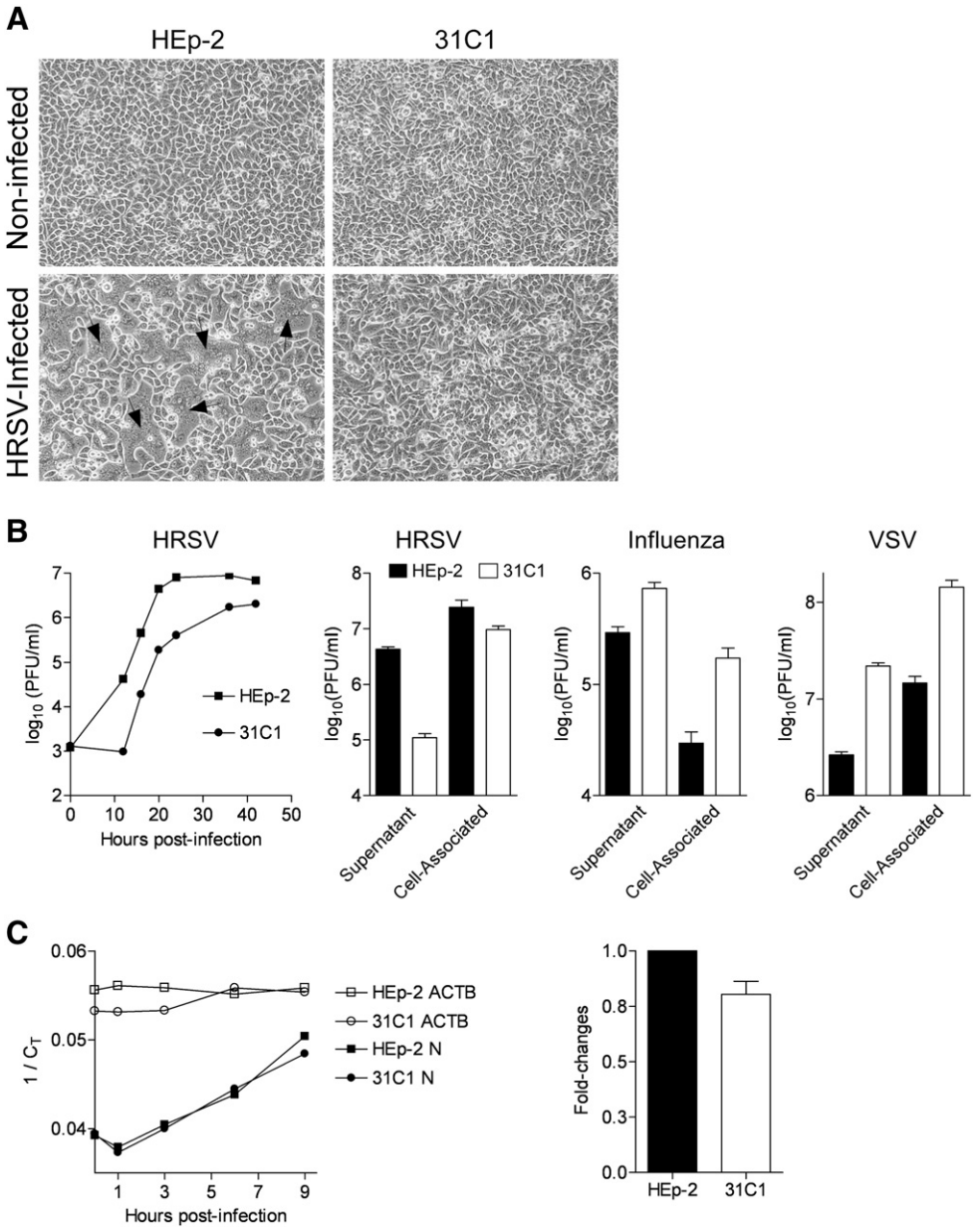


**Fig. 1.** (A) Cytopathic effect of HEp-2 and 31C1 cell cultures infected with HRSV (Long strain). Cells were either uninfected (top panels) or infected with HRSV at a MOI of 1 pfu/cell and photographed at 24 h p.i. Note the presence of syncytia in infected HEp-2 cells (arrows), which were absent from the infected 31C1 culture. (B) Virus growth after infection of HEp-2 and 31C1 cells with HRSV at a MOI of 3 pfu/cell. Virus titer was determined by plaque assay with a mixture of anti-G and anti-F antibodies in the clarified (5000×g/10 min) supernatant of infected cells. Virus yields in the culture supernatant and associated with cells were compared at 20 h p.i. For cell-associated virus, cells were washed with DMEM2, scrapped off in fresh DMEM2, disaggregated by thoroughly pippeting and brief sonication in an ultrasonic bath, and virus titrated in the clarified supernatant. Similar experiments were carried out for influenza and VSV viruses, except that influenza infections were done in the absence of FCS and VSV titration was done at 6 h p.i. Mean and standard deviation of three experiments are shown. (C) Left panel: Accumulation of HRSV nucleoprotein RNA (N) in infected HEp-2 and 31C1 cells (MOI of 3 pfu/cell) estimated by quantitative RT-PCR. Right panel: Relative accumulation of N RNA in 31C1 cells compared with HEp-2 cells at 20 h p.i. using β-actin (ACTB) as endogenous control (the mean and standard deviation of three experiments are shown). Threshold cycle ($C_T$) is the PCR cycle number at which the fluorescence signal meets the threshold in the amplification plot.

HRSV, but were much less obvious after infection of 31C1 cells (Fig. 4B). As already reported (Casola et al., 2000; Jamaluddin et al., 1998), induction of c-Jun represents an early event after HRSV infection of HEp-2 cells, whereas degradation of IKBα occurs at later times (Fig. 4B).

In agreement with the low expression of PRRs and the low activation of transcription factors involved in the innate immune response, 31C1 cells secreted lower amounts of several cytokines into the culture supernatant after virus infection than HEp-2 cells (Fig. 4C). This was more evident for CXCL8 (IL-8), IL-6 and CCL5 (RANTES) that were expressed four, thirty four and one hundred and twenty two times less, respectively, in 31C1 than in HEp-2 cells after HRSV infection (20 h p.i.). In all cases, basal expression, corresponding to non-infected cells, was also lower in 31C1 than in HEp-2 cells (Fig. 4C).

The induction of IFN-β was also tested in infected HEp-2 and 31C1 cells, but no significant induction of this cytokine was observed in either cell type (not shown). This might be related to the ability of HRSV to block the activation of IFN-β transcription (Ling et al., 2009; Spann et al., 2005; Swedan et al., 2009). It is generally accepted that IFN-β induction is triggered by recognition of viral RNAs by PRRs. RIG-I and TLR3 also recognize artificial RNAs, such poly(I:C), leading to type I IFN induction (Baum and Garcia-Sastre). Thus, we decided to test the induction of IFN-β after poly(I:C) stimulation of HEp-2 and

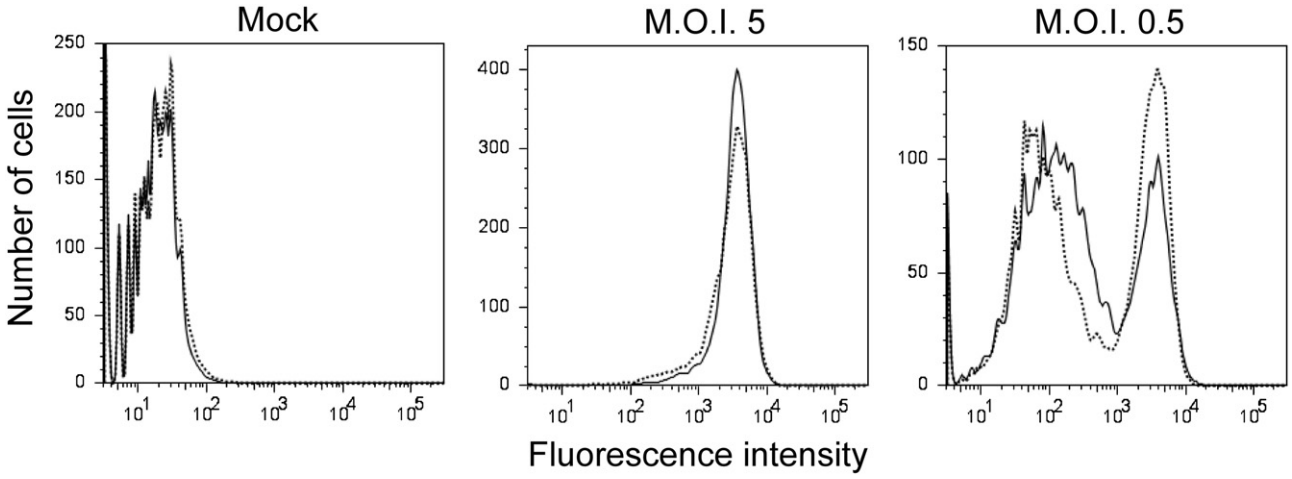


**Fig. 2.** Flow cytometry of HEp-2 and 31C1 cells. Cells were either infected at MOI of 5 or 0.5 pfu/cell, or left uninfected (mock). Twenty hours later, the cells were resuspended, stained with a mixture of anti-F (47F) and anti-G (021/1G) monoclonal antibodies and the fluorescence was measured by flow cytometry. Dotted lines correspond to 31C1 cells and continuous lines to HEp-2 cells.

31C1 cells. Fig. 4D shows that a significant induction of IFN-β was observed only in HEp-2 cells following poly(I:C) transfection with DEAE-Dextran, but not in 31C1 cells.

From the above experiments, it became apparent that the initial innate immune response triggered by virus infection and mediated by PRRs was impaired in 31C1 cells. This response usually leads to the synthesis of interferon that, in turn, induces the production of many antiviral products (Randall and Goodbourn, 2008). To test whether or not 31C1 cells were also deficient in the induction of this second wave of the cellular innate immune response, expression of RIG-I, an interferon stimulated gene that is instrumental in triggering the innate immune response to HRSV, was analyzed. HRSV infection enhanced expression of this gene about seven times in HEp-2 cells, but not in 31C1 cells (Fig. 5, HRSV). Furthermore, treatment of HEp-2 cells with IFN-β also enhanced the expression of RIG-I about eleven times, but in contrast to HRSV infection, this treatment also enhanced the expression of RIG-I in 31C1 cells by more than fifty times (Fig. 5, IFN-β). Therefore, 31C1 cells were capable of responding to exogenous IFN-β. Note that the levels of mRNAs represented in Fig. 5 after IFN-β stimulation are relative to the corresponding non-treated cells, HEp-2 or 31C1. When a direct comparison between HEp-2 and 31C1 IFN-β stimulated cells was carried out, the relative levels of RIG-I were about three times higher in HEp-2 cells than in 31C1 cells (not shown). This is because of the very low expression of basal RIG-I in 31C1 cells.

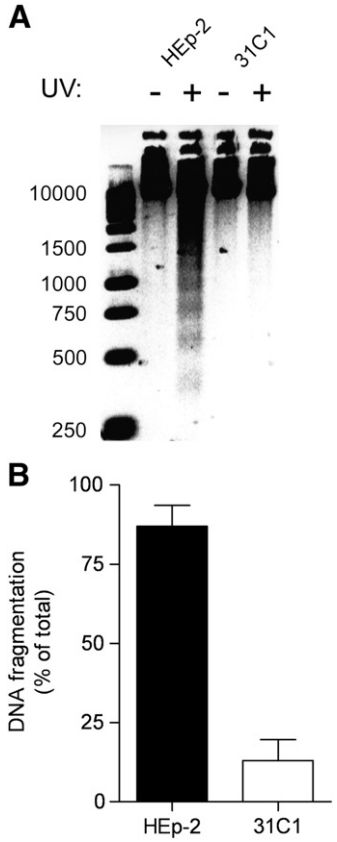


**Fig. 3.** DNA fragmentation in cells irradiated with UV light. (A) Cells were irradiated with UV light for 5 min, incubated for 3 h at 37 °C, and 3 μg of extracted DNA were loaded into a 2% agarose gel. (B) Alternatively, DNA fragmentation was quantified by ELISA in samples of three independent experiments (see Materials and methods).

Finally, an important question was whether additional clones with the same properties of 31C1 cells could be obtained from the persistently infected culture from which 31C1 derived, as well as from uninfected HEp-2 cells. To consider this question, five cell clones were isolated from uninfected HEp-2 cells by limiting dilution, and basal expression of TLR3 and RIG-I mRNA was examined along with five clones derived from passage four and fifteen of the HRSV persistently infected cells (Martinez et al., 2009). Basal expression of TLR3 and RIG-I in the five clones derived from uninfected cells varied between approximately half and twice the value found in uncloned HEp-2 cells, but never reached the extremely low values found in the clones derived from the persistently infected culture, especially after the fifteenth passage (Fig. 6).

## Discussion

In a previous study, we established a culture of HEp-2 cells persistently infected with HRSV, from the few cells that survived a lytic infection. Analysis of the persistently infected culture showed great heterogeneity between cells at the level of viral antigen expression, ranging from negative to high positivity as measured by flow cytometry (Martinez et al., 2009). To simplify the study, cells from the persistent culture were cloned at different passages (fourth, fifteenth and thirty-first). In all cases, the cell clones were "cured" of HRSV, however they were still susceptible to virus infection (Martinez et al., 2009). In the present study we have examined more in depth the characteristics of one of these cell clones, obtained after thirty-one weekly passages of the persistently infected culture. This clone (31C1) did not produce either infectious virus or viral antigens, and no viral RNA was detected. HRSV infected and replicated in 31C1 cells as

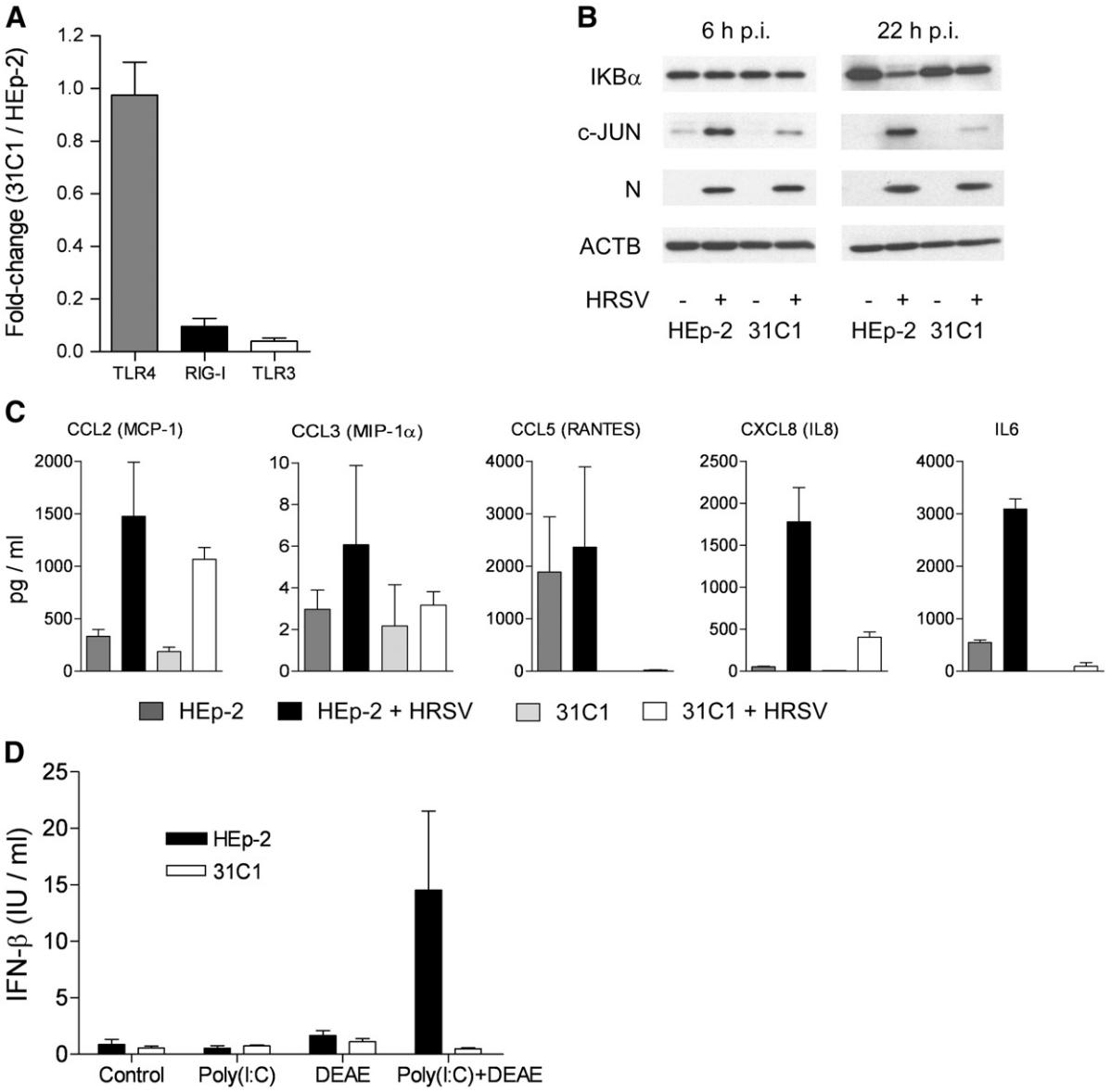


**Fig. 4.** Reduced innate immune response in 31C1 cells. (A) Relative expression of TLR4, RIG-I and TLR3 mRNA in 31C1 compared with HEp-2 cells. Quantitative RT-PCR was performed for the indicated genes in uninfected 31C1 and HEp-2 cells and compared using the gene of ACTB (β-actin) as endogenous control. Data represent the mean and standard deviation from three experiments. (B) Degradation of IKBα and expression of c-Jun in 31C1 and HEp-2 infected cells (MOI of 3 pfu/cell). Ten micrograms of total proteins from the corresponding cell extracts were fractionated by SDS-PAGE and visualized by Western blotting with specific antibodies. An antibody against β-actin (ACTB) was included as an internal control. The amount of viral nucleoprotein (N) was assessed with a specific monoclonal antibody (021/2N) as a control of the infection. (C) Accumulation of cytokines in the supernatant of 31C1 and HEp-2 cells after 20 h of infection with HRSV (MOI of 3 pfu/cell), as determined by solid phase sandwich ELISA. Mean and standard deviations of three independent experiments are shown. (D) Induction of IFN-β in poly(I:C) treated HEp-2 and 31C1 cells. HEp-2 and 31C1 cells were incubated for 30 h in the presence of 10 μg/ml poly (I:C) alone, 10 μg/ml DEAE-Dextran alone, a mixture of both or left untreated (control). IFN-β was measured in the supernatant by ELISA. Mean and standard deviations of three independent experiments are shown.

efficiently as in the parental HEp-2 cells, but syncytia formation and virus release were impaired. In addition, 31C1 cells were resistant to apoptosis, and responded to HRSV infection with a weak innate immune response.

Analyses of cell clones derived from cultures persistently infected with other viruses have contributed to understanding viral and/or cellular processes relevant for virus infection, such as viral attachment and entry (Borzakian et al., 1992; Dermody et al., 1993; Kaplan et al., 1989; Mrukowicz et al., 1998; Zhong et al., 2006). In 31C1 cells, HRSV attachment, entry, and replication were apparently unaffected, but release of virus to the culture supernatant was diminished when compared with the parental HEp-2 cells, a characteristic apparently specific for HRSV infection, since it was not observed with other viruses such as influenza or VSV. This limitation in HRSV release, together with a clear inhibition of syncytia formation, led to a decreased spreading of HRSV in 31C1 infected cells. This may have favored cell survival in the original persistently infected culture, although additional viral and/or cellular characteristics might have also played a role, since HRSV infection of 31C1 cultures eventually led to cell death. The reason for the low release of virus in 31C1 cells is unknown, although possible mechanisms are currently under investigation. For example, cholesterol biosynthesis is required for assembly and budding of several viruses, including HRSV (Robinzon et al., 2009; Yeo et al., 2009), therefore differences in cholesterol biosynthesis may exist between HEp-2 and 31C1 cells. Proteins of the host machinery involved in HRSV budding, such as those of the Rab11 family, are also necessary for virus release (Utley et al., 2008). Differences in the composition and/or amount of plasma membrane

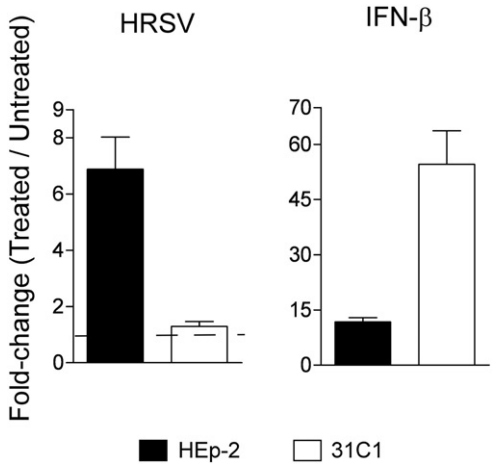


**Fig. 5.** RIG-I expression in cells infected with HRSV (MOI of 3 pfu/cell, 20 h p.i.) or treated with interferon-β (500 U/ml, 3 h after the treatment). The amount of RIG-I RNA was determined by quantitative RT-PCR and compared with uninfected (HRSV) or non-treated (IFN-β) cells using ACTB as an endogenous control. Data represent the mean and standard deviation from three experiments. Fold-change equal to 1 (no changes in mRNA expression between uninfected and infected cells) is represented by a dashed line.

glycosaminoglycans, molecules to which HRSV binds (Hallak et al., 2000; Krusat and Streckert, 1997; Martinez and Melero, 2000), may contribute to retaining the virus at the cell surface, given that HRSV lacks a receptor-destroying activity to liberate progeny virions from the plasma membrane. Finally, differences in the phosphorylation of HRSV phosphoprotein (P) in HEp-2 and 31C1 cells may account for differences in virus release, since it has been described that mutations of the serines at several phosphorylation sites of the HRSV P substantially increase the amount of cell-associated virus (Lu et al., 2002).

It has been reported that HRSV induces cell apoptosis at late stages of infection (Monick et al., 2005; O'Donnell et al., 1999). Therefore, it is relevant that 31C1 cells are partly resistant to apoptosis. This characteristic should facilitate cell survival but, at the same time, would support virus production for a longer time in the infected cell. Thus, an equilibrium may be reached that benefits both the virus and the cell while establishment of the persistent infection. In other virus–cell systems it has been found that inhibition of apoptosis facilitates, among others, persistent infections of HIV-1 and hepatitis C virus (Antoni et al., 1995; Mankouri et al., 2009), and it has been described that cells cured of a persistent poliovirus infection showed reduced apoptosis (Labadie et al., 2007).

It was observed that the innate immune response of 31C1 cells to infection with HRSV was defective. However, it is important to note that 31C1 cells do not show a generalized impairment of the innate immune response, since they responded to IFN-β when added exogenously. Instead, a very early and specific step involved in the detection of virus infection seems to be affected. The innate immune response in infected cells is initiated by pattern recognition receptors such as toll-like receptors (TLRs), retinoic acid-inducible protein I (RIG-I) and melanoma differentiation-associated protein-5 (Mda5) (Kawai and Akira, 2008; Randall and Goodbourn, 2008). Specifically, TL3, and RIG-I have a central role in the recognition of HRSV RNA in infected epithelial cells (Liu et al., 2007; Rudd et al., 2005). This recognition activates the transcription factors IRF3, NF-κB, and AP-1, leading to the induction of cytokine production (Kawai and Akira, 2008). Thus, it is likely that the very low expression levels of TLR3 and RIG-I in uninfected 31C1 cells were responsible for the weak activation of NF-κB and c-JUN (AP-1), and subsequent low production of CCL5 (RANTES), CXCL8 (IL-8) and IL-6, in 31C1 infected cells, a hypothesis supported by the lack of 31C1 cells response to poly(I:C) stimulation. A marked suppression of IL-8, RANTES, TNF-α and IL-6 production and NF-κB activation after lipopolysaccharide stimulation of monocytic cells persistently infected with measles virus has been reported recently, although no significant differences in the level of expression of TLRs were found (Indoh et al., 2007). The very low expression of PRRs in 31C1 cells was not observed in clones derived from uninfected HEp-2 cells, but it was observed in all clones selected at different passages of the persistently infected culture from which 31C1 cells were obtained, strongly indicating that this characteristic was acquired because of the selective pressure of HRSV infection. Cell variants with different modifications have also been selected from other virus–cell systems (Ahmed et al., 1981; Chen and Baric, 1996; de la Torre et al., 1989, 1988; Dermody et al., 1993; Martin Hernandez et al., 1994; Mrukowicz et al., 1998; Ron and Tal, 1985; Zhong et al., 2006).

The possibility that airway epithelial cell variants similar to 31C1 cells may also arise *in vivo* should be considered. The rapid renewal of the airway epithelium, especially after injury (Crystal et al., 2008; Heguy et al., 2007), would provide the cellular substrate on which HRSV infection may exert a selective pressure. Selection of cell variants that support HRSV replication for long time (i.e. resistant to apoptosis and/or with an impaired innate immune response to the virus) may occur in an environment of continuous cell renewal and replicating virus. This scenario may be similar to that described for some chronic respiratory pathologies, such as chronic obstructive pulmonary disease (COPD), in which HRSV RNA has been detected during extended periods of time in the stable phase of the disease (Sikkel et al., 2008; Wilkinson et al., 2006). Thus, 31C1 cells may be a useful "in vitro" model system for studies on the interaction of HRSV and respiratory epithelial cells "in vivo", although some caveats must be taken into account because of the additional complexity of the "in vivo" situation.

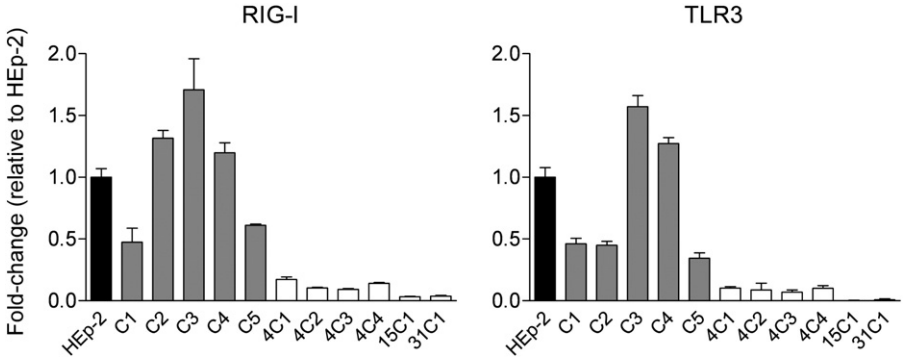


**Fig. 6.** Expression of RIG-I and TLR3 in uncloned HEp-2 cells and clones derived from them (grey bars) and from the persistently infected culture (white bars). Quantitative RT-PCR was used to determine the amount of mRNA in each culture. Relative expression to uncloned HEp-2 was calculated in triplicate using ACTB as endogenous control. The clones derived from the persistently infected HEp-2 culture were obtained after passage 4 (4C1, 4C2, 4C3 and 4C4), passage 15 (15C1) or passage 31 (31C1).

## Materials and methods

### Cells and viruses

The human HEp-2 and canine MDCK epithelial cell lines, and the baby hamster kidney (BHK-21) cells were grown in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% fetal calf serum (FCS) (Linus), 4 mM glutamine (Lonza), 100 U/ml penicillin (Lonza) and 100 U/ml streptomycin (Lonza) (DMEM10) at 37 °C in 5% $CO_2$. The Long strain of HRSV was propagated in HEp-2 cells in DMEM with 2% FCS, glutamine and antibiotics (DMEM2). Viruses were purified from clarified culture supernatants by polyethylene glycol precipitation and centrifugation in a 30–45–60% discontinuous sucrose gradient, and titrated by plaque assay in HEp-2 cells as previously described (Martinez et al., 2007). HEp-2 cells persistently infected with the Long strain of HRSV were obtained by culture of cells that survived a lytic virus infection in DMEM10 at 37 °C in 5% $CO_2$ and passaged weekly at 1/3 dilution (Martinez et al., 2009). Persistently infected cells were cloned at passage thirty-one by two rounds of limiting dilution to obtain 31C1 cells: In brief, cells were trypsinized and serial two-fold dilutions were plated in 96-well microplates. Wells were monitored daily for cell growth and those at the highest dilution that showed individual clones were trypsinized again and replated as before. After this second round of cloning, cells in the selected wells (those from the highest dilution showing a single clone) were trypsinized and expanded. The A/Victoria/3/75 strain of influenza virus was propagated in MDCK cells in the absence of FCS and the presence of 25 μg/ml of trypsin. Vesicular stomatitis virus (Indiana serotype) was grown in BHK-21 cells. Virus titers were determined by plaque assay in MDCK (influenza) or BHK-21 cells (VSV) layered with 0.7% low-melting point agarose (Conda).

### Antibodies

Antibodies against G (021/1G, 021/2G), F (47F), N (021/N, 79N) and P (67P) proteins of HRSV have been described previously (Garcia-Barreno et al., 1989; Martinez et al., 1997). Mouse monoclonal against beta actin (ab8224), rabbit monoclonals against c-JUN (ab32137) and IKB alpha (ab32518), and goat polyclonal to rabbit IgG, horseradish peroxidase linked whole antibody, were obtained from Abcam (UK). Anti-mouse IgG, horseradish peroxidase linked whole antibody (from sheep) was purchased from GE Healthcare. Goat anti-mouse IgM + IgG + IgA (H + L) R-phycoerythrin conjugate was obtained from SouthernBiotech.

### Quantitative RT-PCR and Western blots

HEp-2 and 31C1 cells were mock-infected or infected with purified virus (Long strain) in DMEM2. After 90 min of adsorption, fresh medium was added and cells were incubated at 37 °C. Total RNA was extracted with TRIZOL reagent (Invitrogen) and further purified with the RNeasy kit (Qiagen) at the post-infection (p.i.) time indicated in the figure legends. RNA was reverse-transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems) and amplified using TaqMan quantitative gene expression assays (Applied Biosystems). PCR primers and TaqMan MGB probes (FAM dye-labeled) for the following genes were products from Assays-on-demand (Applied Biosystems): ACTB (Hs99999903_m1), TLR4 (Hs00152939_m1), TLR3 (Hs01551078_m1) and DDX58 (RIG-I) (Hs00204833_m1). PCRs were performed in triplicate following the manufacturer´s protocols on a StepOne sequence detection system (Applied Biosystems). ACTB (β-actin) cDNA was used as an endogenous control and the comparative $C_T$ ($\Delta\Delta C_T$) method was used for relative quantifications (Applied Biosystems User Bulletin no.2). A Custom TaqMan Gene Expression Assay (Applied Biosystems) was used for amplification and quantification of the nucleoprotein RNA of HRSV (forward primer: 5´CATGATTCTCCTGATTGTGGGATGA 3´, reverse primer: 5´ TCACGGCTGTAAGACCAGATCTAT 3´, probe: 5´CCCCTGCTGCCAATTT3´).

In some experiments, protein cell extracts were prepared from cultures infected under the same conditions as those used for RNA extraction. Proteins were separated by SDS-PAGE and analyzed by Western blotting with the antibodies indicated in each figure.

### Flow cytometry

Cells were detached with PBS-10 mM EDTA, incubated for 20 min at 4 °C with a mixture of 021/2G and 47F antibodies, washed with PBS-2% FCS, incubated for another 20 min with the secondary antibody (goat anti-mouse IgM + IgG + IgA (H + L) R-phycoerythrin conjugate), washed and fixed with 1% paraformaldehyde. Sample fluorescence was measured on a FACS-Canto flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo 7.5.2. software (TreeStar, San Carlos, CA).

### DNA fragmentation assay

Cells were irradiated with UV light for 5 min in PBS. After this period, PBS was aspirated, fresh medium was added, and the cells were incubated for 3 h at 37 °C. Following the incubation period, cells were detached with PBS-1 mM EDTA and DNA was extracted with the Easy-DNA kit (Invitrogen). Three micrograms of DNA were loaded onto a 2% agarose gel and visualized with GelRed Nucleic Acid Gel Stain (Biotium). Alternatively, DNA fragmentation was determined by Cell Death Detection ELISA (Roche), a photometric enzyme immunoassay for detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death.

### ELISA assays for cytokines

Semiconfluent 90 mm dishes of HEp-2 and 31C1 cells were mock-infected or infected with the Long strain of HRSV in DMEM2 at a MOI of 3 pfu/cell. Twenty hours later, the amount of the chemokines CCL2/MCP-1, CCL3/MIP-1α, CCL5/RANTES and the interleukins IL-6 and IL-8 was determined in the clarified supernatant by solid phase sandwich ELISA kits, following manufacturer protocols (Invitrogen, KHC1011, KAC2201, KHC1031, KAC1261 and KHC0081).

### Poly(I:C) treatment

Semiconfluent HEp-2 and 31C1 cells growing in 6-wells plates were treated with either 10 μg/ml of poly(I:C) (Sigma-Aldrich, P9582), 10 μg/ml of DEAE-Dextran (Sigma-Aldrich, D9885) or 10 μg/ml of poly (I:C) plus 10 μg/ml of DEAE-Dextran for 30 h. After this time, the amount of IFN-β was measured in the clarified culture supernatant by ELISA (Invitrogen, KAC1201).

## Acknowledgments

This work was supported by grants UIPY 1469/07 from “Instituto de Salud Carlos III” and PI080702 from “Fondo de Investigación Sanitaria” to IM and by grant SAF2009-11632 from “Ministerio de Ciencia e Innovación” to JAM. The “Unidad de Biología Viral” is part of the VIRHOST consortium funded by “Comunidad de Madrid”.

We would like to thank Joanna Rawling for English corrections.

## References

Ahmed, R., Canning, W.M., Kauffman, R.S., Sharpe, A.H., Hallum, J.V., Fields, B.N., 1981. Role of the host cell in persistent viral infection: coevolution of L cells and reovoirus during persistent infection. Cell 25, 325–332.

Antoni, B.A., Sabbatini, P., Rabson, A.B., White, E., 1995. Inhibition of apoptosis in human immunodeficiency virus-infected cells enhances virus production and facilitates persistent infection. J. Virol. 69, 2384–2392.

Bachi, T., 1988. Direct observation of the budding and fusion of an enveloped virus by video microscopy of viable cells. J. Cell Biol. 107, 1689–1695.

Baum, A., Garcia-Sastre, A., 2010. Induction of type I interferon by RNA viruses: cellular receptors and their substrates. Amino Acids 38, 1283–1299.
Borzakian, S., Couderc, T., Barbier, Y., Attal, G., Pelletier, I., Colbere-Garapin, F., 1992. Persistent poliovirus infection: establishment and maintenance involve distinct mechanisms. Virology 186, 398–408.
Casola, A., Garofalo, R.P., Jamaluddin, M., Vlahopoulos, S., Brasier, A.R., 2000. Requirement of a novel upstream response element in respiratory syncytial virus-induced IL-8 gene expression. J. Immunol. 164, 5944–5951.
Collins, P., Crowe, J., 2007. Respiratory syncytial virus and metapneumovirus, In: Knipe, D., Howley, P. (Eds.), Fields Virology, Fifth ed. Lippincott Williams & Wilkins, Philadelphia, pp. 1601–1646.
Collins, P.L., Graham, B.S., 2008. Viral and host factors in human respiratory syncytial virus pathogenesis. J. Virol. 82, 2040–2055.
Crystal, R.G., Randell, S.H., Engelhardt, J.F., Voynow, J., Sunday, M.E., 2008. Airway epithelial cells: current concepts and challenges. Proc. Am. Thorac. Soc. 5, 772–777.
Chen, W., Baric, R.S., 1996. Molecular anatomy of mouse hepatitis virus persistence: coevolution of increased host cell resistance and virus virulence. J. Virol. 70, 3947–3960.
de la Torre, J.C., Martinez-Salas, E., Diez, J., Domingo, E., 1989. Extensive cell heterogeneity during persistent infection with foot-and-mouth disease virus. J. Virol. 63, 59–63.
de la Torre, J.C., Martinez-Salas, E., Diez, J., Villaverde, A., Gebauer, F., Rocha, E., Davila, M., Domingo, E., 1988. Coevolution of cells and viruses in a persistent infection of foot-and-mouth disease virus in cell culture. J. Virol. 62, 2050–2058.
Dermody, T.S., Nibert, M.L., Wetzel, J.D., Tong, X., Fields, B.N., 1993. Cells and viruses with mutations affecting viral entry are selected during persistent infections of L cells with mammalian reoviruses. J. Virol. 67, 2055–2063.
Garcia-Barreno, B., Palomo, C., Penas, C., Delgado, T., Perez-Brena, P., Melero, J.A., 1989. Marked differences in the antigenic structure of human respiratory syncytial virus F and G glycoproteins. J. Virol. 63, 925–932.
Hallak, L.K., Spillmann, D., Collins, P.L., Peeples, M.E., 2000. Glycosaminoglycan sulfation requirements for respiratory syncytial virus infection. J. Virol. 74, 10508–10513.
Hegele, R.G., Hayashi, S., Bramley, A.M., Hogg, J.C., 1994. Persistence of respiratory syncytial virus genome and protein after acute bronchiolitis in guinea pigs. Chest 105, 1848–1854.
Heguy, A., Harvey, B.G., Leopold, P.L., Dolgalev, I., Raman, T., Crystal, R.G., 2007. Responses of the human airway epithelium transcriptome to in vivo injury. Physiol. Genomics 29, 139–148.
Indoh, T., Yokota, S., Okabayashi, T., Yokosawa, N., Fujii, N., 2007. Suppression of NF-kappaB and AP-1 activation in monocytic cells persistently infected with measles virus. Virology 361, 294–303.
Jamaluddin, M., Casola, A., Garofalo, R.P., Han, Y., Elliott, T., Ogra, P.L., Brasier, A.R., 1998. The major component of IkappaBalpha proteolysis occurs independently of the proteasome pathway in respiratory syncytial virus-infected pulmonary epithelial cells. J. Virol. 72, 4849–4857.
Kapikian, A.Z., Mitchell, R.H., Chanock, R.M., Shvedoff, R.A., Stewart, C.E., 1969. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol. 89, 405–421.
Kaplan, G., Levy, A., Racaniello, V.R., 1989. Isolation and characterization of HeLa cell lines blocked at different steps in the poliovirus life cycle. J. Virol. 63, 43–51.
Kawai, T., Akira, S., 2008. Toll-like receptor and RIG-I-like receptor signaling. Ann. NY Acad. Sci. 1143, 1–20.
Klein Klouwenberg, P., Tan, L., Werkman, W., van Bleek, G.M., Coenjaerts, F., 2009. The role of Toll-like receptors in regulating the immune response against respiratory syncytial virus. Crit. Rev. Immunol. 29, 531–550.
Krishnan, S., Halonen, M., Welliver, R.C., 2004. Innate immune responses in respiratory syncytial virus infections. Viral Immunol. 17, 220–233.
Krusat, T., Streckert, H.J., 1997. Heparin-dependent attachment of respiratory syncytial virus (RSV) to host cells. Arch. Virol. 142, 1247–1254.
Labadie, K., Saulnier, A., Martin-Latil, S., Colbere-Garapin, F., 2007. Reduced apoptosis in human intestinal cells cured of persistent poliovirus infection. J. Virol. 81, 3033–3036.
Levine, S., Hamilton, R., 1969. Kinetics of the respiratory syncytial virus growth cycle in HeLa cells. Arch. Gesamte Virusforsch. 28, 122–132.
Ling, Z., Tran, K.C., Teng, M.N., 2009. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. J. Virol. 83, 3734–3742.
Liu, P., Jamaluddin, M., Li, K., Garofalo, R.P., Casola, A., Brasier, A.R., 2007. Retinoic acid-inducible gene I mediates early antiviral response and Toll-like receptor 3 expression in respiratory syncytial virus-infected airway epithelial cells. J. Virol. 81, 1401–1411.
Lu, B., Ma, C.H., Brazas, R., Jin, H., 2002. The major phosphorylation sites of the respiratory syncytial virus phosphoprotein are dispensable for virus replication in vitro. J. Virol. 76, 10776–10784.
Mankouri, J., Dallas, M.L., Hughes, M.E., Griffin, S.D., Macdonald, A., Peers, C., Harris, M., 2009. Suppression of a pro-apoptotic K+ channel as a mechanism for hepatitis C virus persistence. Proc. Natl Acad. Sci. USA 106, 15903–15908.
Martin Hernandez, A.M., Carrillo, E.C., Sevilla, N., Domingo, E., 1994. Rapid cell variation can determine the establishment of a persistent viral infection. Proc. Natl Acad. Sci. USA 91, 3705–3709.
Martinez, I., Dopazo, J., Melero, J.A., 1997. Antigenic structure of the human respiratory syncytial virus G glycoprotein and relevance of hypermutation events for the generation of antigenic variants. J. Gen. Virol. 78 (Pt 10), 2419–2429.
Martinez, I., Lombardia, L., Garcia-Barreno, B., Dominguez, O., Melero, J.A., 2007. Distinct gene subsets are induced at different time points after human respiratory syncytial virus infection of A549 cells. J. Gen. Virol. 88, 570–581.
Martinez, I., Lombardia, L., Herranz, C., Garcia-Barreno, B., Dominguez, O., Melero, J.A., 2009. Cultures of HEp-2 cells persistently infected by human respiratory syncytial virus differ in chemokine expression and resistance to apoptosis as compared to lytic infections of the same cell type. Virology 388, 31–41.
Martinez, I., Melero, J.A., 2000. Binding of human respiratory syncytial virus to cells: implication of sulfated cell surface proteoglycans. J. Gen. Virol. 81, 2715–2722.
Monick, M.M., Cameron, K., Staber, J., Powers, L.S., Yarovinsky, T.O., Koland, J.G., Hunninghake, G.W., 2005. Activation of the epidermal growth factor receptor by respiratory syncytial virus results in increased inflammation and delayed apoptosis. J. Biol. Chem. 280, 2147–2158.
Mrukowicz, J.Z., Wetzel, J.D., Goral, M.I., Fogo, A.B., Wright, P.F., Dermody, T.S., 1998. Viruses and cells with mutations affecting viral entry are selected during persistent rotavirus infections of MA104 cells. J. Virol. 72, 3088–3097.
O'Donnell, D.R., Milligan, L., Stark, J.M., 1999. Induction of CD95 (Fas) and apoptosis in respiratory epithelial cell cultures following respiratory syncytial virus infection. Virology 257, 198–207.
Oshansky, C.M., Zhang, W., Moore, E., Tripp, R.A., 2009. The host response and molecular pathogenesis associated with respiratory syncytial virus infection. Future Microbiol. 4, 279–297.
Perez-Yarza, E.G., Moreno, A., Lazaro, P., Mejias, A., Ramilo, O., 2007. The association between respiratory syncytial virus infection and the development of childhood asthma: a systematic review of the literature. Pediatr. Infect. Dis. J. 26, 733–739.
Prince, G.A., Curtis, S.J., Yim, K.C., Porter, D.D., 2001. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. J. Gen. Virol. 82, 2881–2888.
Randall, R.E., Goodbourn, S., 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. 89, 1–47.
Robinzon, S., Dafa-Berger, A., Dyer, M.D., Paeper, B., Proll, S.C., Teal, T.H., Rom, S., Fishman, D., Rager-Zisman, B., Katze, M.G., 2009. Impaired cholesterol biosynthesis in a neuronal cell line persistently infected with measles virus. J. Virol. 83, 5495–5504.
Ron, D., Tal, J., 1985. Coevolution of cells and virus as a mechanism for the persistence of lymphotropic minute virus of mice in L-cells. J. Virol. 55, 424–430.
Rudd, B.D., Burstein, E., Duckett, C.S., Li, X., Lukacs, N.W., 2005. Differential role for TLR3 in respiratory syncytial virus-induced chemokine expression. J. Virol. 79, 3350–3357.
Schwarze, J., O'Donnell, D.R., Rohwedder, A., Openshaw, P.J., 2004. Latency and persistence of respiratory syncytial virus despite T cell immunity. Am. J. Respir. Crit. Care Med. 169, 801–805.
Sikkel, M.B., Quint, J.K., Mallia, P., Wedzicha, J.A., Johnston, S.L., 2008. Respiratory syncytial virus persistence in chronic obstructive pulmonary disease. Pediatr. Infect. Dis. J. 27, S63–S70.
Spann, K.M., Tran, K.C., Collins, P.L., 2005. Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. J. Virol. 79, 5353–5362.
Streckert, H.J., Philippou, S., Riedel, F., 1996. Detection of respiratory syncytial virus (RSV) antigen in the lungs of guinea pigs 6 weeks after experimental infection and despite of the production of neutralizing antibodies. Arch. Virol. 141, 401–410.
Swedan, S., Musiyenko, A., Barik, S., 2009. Respiratory syncytial virus nonstructural proteins decrease levels of multiple members of the cellular interferon pathways. J. Virol. 83, 9682–9693.
Utley, T.J., Ducharme, N.A., Varthakavi, V., Shepherd, B.E., Santangelo, P.J., Lindquist, M. E., Goldenring, J.R., Crowe Jr., J.E., 2008. Respiratory syncytial virus uses a Vps4-independent budding mechanism controlled by Rab11-FIP2. Proc. Natl Acad. Sci. USA 105, 10209–10214.
Wilkinson, T.M., Donaldson, G.C., Johnston, S.L., Openshaw, P.J., Wedzicha, J.A., 2006. Respiratory syncytial virus, airway inflammation, and FEV1 decline in patients with chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 173, 871–876.
Yeo, D.S., Chan, R., Brown, G., Ying, L., Sutejo, R., Aitken, J., Tan, B.H., Wenk, M.R., Sugrue, R.J., 2009. Evidence that selective changes in the lipid composition of raft-membranes occur during respiratory syncytial virus infection. Virology 386, 168–182.
Zhang, Y., Luxon, B.A., Casola, A., Garofalo, R.P., Jamaluddin, M., Brasier, A.R., 2001. Expression of respiratory syncytial virus-induced chemokine gene networks in lower airway epithelial cells revealed by cDNA microarrays. J. Virol. 75, 9044–9058.
Zhong, J., Gastaminza, P., Chung, J., Stamataki, Z., Isogawa, M., Cheng, G., McKeating, J.A., Chisari, F.V., 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. J. Virol. 80, 11082–11093.